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Integration into the Phage Attachment Site, *attB*, Impairs Multicellular Differentiation in *Stigmatella aurantiaca*

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Stigmatella aurantiaca **displays a complex developmental life cycle in response to starvation conditions that results in the formation of tree-like fruiting bodies capable of producing spores. The phage Mx8, first isolated from the close relative *Myxococcus xanthus*, is unable to infect *S. aurantiaca* cells and integrate into the genome. However, plasmids containing Mx8 fragments encoding the integrase and *attP* are able to integrate at the *attB* locus in the *S. aurantiaca* genome by site-specific recombination. After recombination between *attP* and *attB*, the *S. aurantiaca* cells were incapable of building normal fruiting bodies but formed clumps and fungus-like structures characteristic of intermediate stages of development displayed by the wild type. We identified two tRNA genes, *trnD* and *trnV*, encoding $tRNA^{Asp}$ and $tRNA^{Val}$, respectively, composing an operon at the *attB* locus of *S. aurantiaca*. Integration of *attP*-containing plasmids resulted in the incorporation of the t_{Mx8} terminator sequence, in addition to a short sequence of Mx8 DNA downstream of *trnD*. The integrant was unable to process the *trnD* transcript at the normal 3′ processing site and displayed a lower level of expression of the *trnVD* operon. In addition, several developmentally regulated proteins were no longer produced in mutants following insertion at the *attB* locus. We hypothesize that the integration of the t_{Mx8} terminator sequence results in reduced levels of mature $tRNA^{Asp}$ and $tRNA^{Val}$ and that altered protein production during development is thereby responsible for the observed phenotype. The *trnVD* locus thus defines a new developmental checkpoint for *Stigmatella aurantiaca*.**

Stigmatella aurantiaca is a δ-proteobacterium and a member of the myxobacteria, which represent a group of social prokaryotes that progress through a multicellular developmental program in response to starvation (31). Cells move into aggregation centers in response to the pheromone stigmolone (29). Ultimately, fruiting bodies differentiate into stalks bearing several sporangioles at their tops. Each sporangiole contains thousands of myxospores (about 10^5) that are able to germinate under suitable conditions (23, 44). Sporulation of vegetative cells can occur independently from fruiting-body formation as a result of exposure to indole or its derivatives (9). The formation of fruiting bodies is strictly coupled to spatially and temporally regulated synthesis of factors required for the expression of many developmentally regulated genes (17, 18). Inactivation of many of the genes encoding these regulatory factors leads to defects in fruiting-body formation. Throughout the developmental program, specific changes in protein production can be observed (16). Thus, the myxobacteria are excellent model organisms for the study of multicellular development in prokaryotes.

The lack of replicating plasmids within the myxobacterial family has been a limiting factor for genetic analysis. Thus, several different myxophages have been characterized that are able to infect *Myxococcus xanthus* (1, 26) and have been widely used. The myxophage Mx8 integrates into the genome by site-specific recombination, and the prophage is stable upon passage of the *M. xanthus* lysogen through cycles of development and germination (28). The Mx8 *attP* site is contained within the *intP* gene that encodes the integrase for site-specific recombination (24, 25, 43). Plasmids containing the Mx8 *intP-attP* gene have been used to integrate DNA constructs into the *attB* site of *M. xanthus*. The site of integration was found to be within a tRNA operon (24, 43). No developmental phenotype was reported for *M. xanthus* strains containing plasmids integrated into the *attB* site, but the activities of several developmentally regulated promoters (Ω4403, Ω4435, and *devRS*), as well as constitutive promoters (*mgl* and *pilA*), are reduced when expressed from the *M. xanthus attB* site (7, 21, 42).

The Mx8 phage is not capable of infecting *S. aurantiaca* wild-type cells, but plasmids containing the Mx8 *intP-attP* gene can integrate into the *S. aurantiaca attB* site by site-specific recombination with high efficiency (36). In this study, we have identified the core sequence for site-specific recombination in *S. aurantiaca* at the 3′ end of *trnD* encoding $tRNA^{Asp}$. As reported for many prokaryotic tRNA genes, *S. aurantiaca trnD* is arranged together with another tRNA-encoding gene, *trnV*, in an operon. No protein- or rRNA-encoding genes were found in the same operon with these tRNA genes. Furthermore, no specific terminator structures were identified in close proximity to *trnVD*. It is known that prokaryotic tRNA genes are first transcribed into precursor tRNAs that contain 5′ leader and 3′ trailer sequences of various lengths. The precursor tRNA is then processed by an enzyme complex composed of endonucleases and exonucleases to generate mature tRNA (5).

Our results show that integration of plasmids into *S. aurantiaca attB*, and therefore into the *trnD* gene (encoding $tRNA^{Asp}$), results in the alteration of two transcription units, *trnD* and *intP*, as previously described for *M. xanthus* (24, 25). We show

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
E. coli		
XL1-Blue		
MRF′	Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA1 supE*44 *thi-1 recA1 gyrA96 relA1 lac* (F′ *proAB lacI*^{q} *ZΔM*15 Tn*10*) (Tc^r)	Stratagene
S. aurantiaca		
DW4/3-1	Wild type (WT) (Sm^r)	30
SM12	WT; *attB*::pSM28 (Km^r Sm^r)	This study
SM31	WT; *neo*; 87 bp upstream of *trnV* (Km^r Sm^r)	This study
SM32	WT; *trnV*::*neo* (Km^r Sm^r)	This study
SM33	WT; *trnD*::*neo* (Km^r Sm^r)	This study
SM34	WT; *mtaB*::*mtaB-attB-tet* (Km^r Sm^r Tc^r)	This study
SM35	SM12; *attR*::pSM86 (Km^r Sm^r Tc^r)	This study
SM41	WT; *trnVD*::(*trnV*/Δ3′ *trnD*-Δ*trp-lacZ-neo*) (merodiploid for *trnVD*) (Km^r Sm^r)	This study
Plasmids		
Mini-Tn*5lacZ1*	Mini transposon (Ap^r Km^r)	4
pBC KS$^+$	Cloning vector (Cm^r)	Stratagene
pBR322	Cloning vector (Tc^r)	New England Biolabs
pBS9	*fbfA*::Δ*trp-lacZ-neo* in pBS SK$^-$	38
pBS23	pBS SK$^-$; harboring a 2.3-kb *mtaB* fragment (Ap^r)	40
pLJS49	pBR322 derivative containing a 10.5-kb Mx8 fragment (Ap^r Km^r)	21
pSM27	2.9-kb Mx8 *intP-attP* in pBC KS$^+$ (Cm^r)	This study
pSM28	pSM27 with *neo* in the SacI site (Cm^r Km^r)	This study
pSM48	Chromosomal DNA of SM12 KpnI restricted and religated; *attR* (Cm^r Km^r)	This study
pSM49	Chromosomal DNA of SM12 XbaI restricted and religated; *attL* (Cm^r Km^r)	This study
pSM77	1.2-kb *attB* fragment from *S. aurantiaca* in pBC KS$^+$ (Cm^r)	This study
pSM80	pSM77; harboring a *neo* gene 87 bp upstream of *trnV* (Cm^r Km^r)	This study
pSM81	*trnV*::*neo* in pSM77 (Cm^r Km^r)	This study
pSM82	*trnD*::*neo* in pSM77 (Cm^r Km^r)	This study
pSM85	pBS23 with 1.2-kb *attB* fragment (Amp^r)	This study
pSM86	1.2-kb *attB* fragment from *S. aurantiaca* and *tet* gene in pBS23 (Amp^r Tc^r)	This study
pSM96	*trnV* Δ3′ *trnD* in pSM*lacZ* (Cm^r Km^r)	This study
pSM*lacZ*	Δ*trp-lacZ-neo* from pBS9 in pBC SK$^-$ (Cm^r Km^r)	This study
pUC4KIXX	Cloning vector (Ap^r Km^r)	Pharmacia

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Km^r, kanamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance; WT, wild type.

that the downstream region of *trnD* is replaced by the Mx8-specific sequence containing the terminator, t_{Mx8}, and additional DNA encoding *intP*. The integration of plasmids into *attB* impairs fruiting-body formation but not motility. Integrants produce clumps or fungus-like structures characteristic of those that are generated at earlier stages of development by the wild type (44, 46). We were able to restore wild-type development to the mutant by complementing it with a wild-type copy of the *trnVD* locus. Furthermore, insertion into *attB* resulted in decreased expression of *trnVD* and abnormal processing of the 3′ trailer sequence of *trnVD*. Together, these results allow us to conclude that the developmental program cannot be completed following integration into the *attB* locus within *trnD* of *S. aurantiaca*. The *trnVD* operon thus defines a new developmental checkpoint for *S. aurantiaca*. The results lead to the hypothesis that replacement of the natural downstream region of *trnVD* is sufficient to disrupt synthesis of developmentally regulated proteins necessary for stalk production. This hypothesis is supported by the observation that protein production was found to be altered for those mutants with insertions within the *attB* site.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *Stigmatella aurantiaca* wild-type DW4/3-1 cells (30) were grown at 32°C in tryptone medium (1% Bacto Tryptone, 0.2% $MgSO_4$) supplemented with streptomycin sulfate (120 μg/ml) or with kanamycin sulfate (50 μg/ml) or oxytetracycline (7.5 μg/ml). Assays for sporulation, fruiting-body formation, and germination were performed as described previously (14, 39). *Escherichia coli* strains were grown in Luria-Bertani medium at 37°C and supplemented with antibiotics as recommended by the manufacturer (Stratagene). Electroporation of *S. aurantiaca* was performed as previously described (41). Motility was assessed by analyzing individual cells or swarms of cells on hard (1.5%) and soft (0.3%) agar surfaces as previously described for *M. xanthus* (37).

DNA manipulations. Standard genetic techniques for in vitro DNA manipulations and cloning were used (34). Genomic DNA was prepared as described previously (27). Southern blot hybridization and detection were performed using the Biotin-Detection-System. DNA fragments used as probes were labeled using the Biotin-High-Prime system (Roche Diagnostics). DNA sequences were determined by the method of Sanger et al. (35) using plasmid double-stranded DNA and synthetic oligonucleotides. For sequencing, the ABI Prism 377 DNA Sequencer System (Perkin-Elmer Corporation) was used.

RNA isolation and reverse transcription-PCR. Total RNA was isolated from cells during either vegetative growth or development. Vegetative cells were grown in tryptone medium and harvested by centrifugation. For developmental samples, aggregates or fruiting bodies were harvested from 1.5% agar surfaces after 24 h of starvation. Total RNA was extracted using the TRIzol Max Bacterial RNA Isolation kit (Invitrogen). DNA contamination was removed with TURBO DNase (Ambion), and RNA was purified using the RNeasy kit system (QIAGEN). cDNA was synthesized with the SuperScript III First-Strand Synthesis System for reverse transcription-PCR (Invitrogen) using random primers. Equal amounts of RNA (1 μg for vegetative samples and 0.5 μg for developmental samples) were used as templates for reverse transcriptase to generate cDNAs in proportion to the starting material. Specific primers were used for the semiquantitative analysis of *trnD* and *trnV*. PCR products were subjected to agarose gel electrophoresis.

2-D analysis. For the preparation of protein extracts, vegetative cells were grown in tryptone medium and harvested by centrifugation at a density of 2×10^8 cells/ml. For developmental samples, aggregates or fruiting bodies were harvested from a solid agar surface and washed twice with phosphate-buffered saline. The harvested cells were resuspended in 500 μl lysis solution (7 M urea, 2 M thiourea, 4% [wt/vol] CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}, 1% dithiothreitol, 0.5% Servalyt, pH 3 to 10, and Complete EDTA-free protease inhibitor cocktail [Roche]). Cell preparations were disrupted with a Branson sonifier six times for 15 s each time at 4°C and centrifuged at 35,000 × *g* for 30 min. The protein concentration of the supernatant was determined, and samples were stored at −80°C until they were used. Two-dimensional (2-D) electrophoresis with immobilized pH gradients (IPG-Dalt) was carried out according to the method of Görg and colleagues (10–13). (For details, see http://www.weihenstephan.de/blm/deg.) IPG gradients (Bio-Rad) with a pH range from 3 to 10 (17 cm) were run on the PROTEAN IEF Cell (Bio-Rad). Equilibration of the focused IPG strips and 2-D sodium dodecyl sulfate-polyacrylamide gel electrophoresis were done as described previously (13). The 2-D gels were stained either with colloidal Coomassie blue for mass spectrometry analysis (Colloidal Blue staining kit; Invitrogen) or with silver according to the method of Heukeshoven and Dernick (15). Mass spectrometry analysis was done at the biomolecular chemistry facility at the Zentrum für Molekulare Biologie, University of Heidelberg.

Construction of *intP-attP*-containing plasmid pSM28 and mutant SM12. To construct plasmid pSM28, a 2.9-kb Mx8 *intP-attP* fragment from plasmid pLJS49 (21) was amplified using PCR and cloned into the BamHI/EcoRI-restricted plasmid pBC KS(+), resulting in plasmid pSM27. A 1.5-kb *neo* gene from pUC4KIXX (Pharmacia) was cloned into the SacI restriction site of pSM27 to construct plasmid pSM28. Plasmid pSM28 was introduced into the *S. aurantiaca* wild type by electroporation. Kanamycin-resistant clones were obtained, and site-specific recombination generated strain SM12 containing the plasmid in the *attB* site of *S. aurantiaca*. Integrants were confirmed by Southern analysis (data not shown).

Cloning and sequencing of *attL*, *attR*, and *attB* regions. To clone the *attL* and *attR* regions of strain SM12 harboring these host-phage junctions, chromosomal DNA of SM12 was digested with KpnI or XbaI and self-ligated. The ligation mixture was used to transform *E. coli* XL-1 Blue, and kanamycin was used for selection. pSM48 containing the *attR* region and pSM49 containing the *attL* region were isolated from the KpnI- and XbaI-digested chromosomal DNA, respectively. Sequence information from plasmids pSM48 and pSM49 was used to amplify the *attB* locus from the *S. aurantiaca* wild type (accession number DQ320314). The DNA fragment containing *attB* was sequenced and analyzed with the tRNAscan-SE software (22) to screen for tRNA genes.

Construction of strain SM41 harboring a *trnVD-lacZ* fusion. Plasmid pSM*lacZ* contains a promotorless Δ*trp-lacZ* fusion gene from the minitransposon Tn*5lacZ* (4) and a *neo* gene from pUC4KIXX derived from plasmid pBS9 (38). In order to obtain a *trnVD-lacZ* translational fusion, a 700-bp fragment containing 563 bp upstream of *trnV*, the *trnV* gene, and 57 bp of *trnD* was amplified and subcloned into pSM*lacZ*, resulting in pSM96. pSM96 was introduced into wild-type cells by electroporation. Kanamycin-resistant clones were obtained after a single recombination event generated the merodiploid strain SM41 (see Fig. 3A). β-Galactosidase activity in strain SM41 was determined under vegetative and developmental conditions as previously described (38). Cell lysates were prepared by sonication after removal of cell debris by centrifugation at 13,000 × *g* for 10 min. Ten micrograms of total protein in 0.1 ml MOPS (morpholinepropanesulfonic acid) buffer (50 mM MOPS, pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) was mixed with 0.3 ml Na phosphate buffer (10 mM Na phosphate, pH 7, 0.1 M NaCl, 1 mM $MgCl_2$) containing the fluorescent substrate 4-MUG (4-methylumbelliferyl-β-D-galactopyramoside) (10 μg). The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by adding 3 ml of 0.1 M glycine buffer (pH 10.3). β-Galactosidase activity releases 4-methylumbelliferone (4-MU), which is detected fluorometrically following excitation at 360 nm and emission at 450 nm.

Complementation of mutant SM12. In order to complement mutant SM12, we generated a plasmid carrying a wild-type copy of the *trnVD* locus that could integrate at an ectopic site. *mtaB* was chosen for ectopic integration because it is a large gene that encodes a polyketide synthase not involved in development (40). A 2.3-kb EcoRI/HindIII fragment of *mtaB* derived from pBS23 was subcloned into pSM77, resulting in plasmid pSM85. pSM77, and therefore pSM85, contains a 1.2-kb fragment spanning the *trnVD* locus and includes the promoter and trailer sequences as described in Results. A tetracycline resistance gene (*tet*) from pBR322 (New England Biolabs) was amplified by PCR and cloned into the XbaI site of plasmid pSM85, generating pSM86. pSM86 should integrate at *mtaB* due to the large region of homology. This was found to occur when pSM86 was introduced into the wild type, generating SM34. However, pSM86 did not integrate at *mtaB* in mutant SM12 but was found to have integrated at the *trnVD* locus. The site of integration was confirmed by Southern analysis. The most likely explanation is that pSM86 integrated by site-specific recombination into *attR* due to the IntX recombinase that should be present in SM12 cells (see below).

Construction of tRNA mutant strains SM31, SM32, and SM33. Plasmids were constructed and used to obtain *S. aurantiaca* strains that contain *neo* cassettes in *trnD* or *trnV* or in the promoter region of the operon. pSM77, containing the entire *trnVD* locus, was digested with BssHII, BlpI, or NaeI for subcloning the *neo* cassette into the promoter region, *trnV*, or *trnD*, respectively. Restricted pSM77 DNA was treated with Klenow enzyme and ligated to an SmaI-restricted *neo* fragment derived from pUC4KIXX. The resulting plasmids contained a *neo* cassette in the promoter region (pSM80), within the *trnV* gene (pSM81), or within the *trnD* gene (pSM82). pSM80, pSM81, and pSM82 were linearized with ScaI and electroporated into the *S. aurantiaca* wild-type strain. Kanamycin-resistant mutant strains that had undergone double homologous recombination were obtained in which the promoter region (SM31), *trnV* (SM32), or *trnD* (SM33) was disrupted by the *neo* cassette.

RESULTS

Integration of plasmids into the *S. aurantiaca attB* locus impairs fruiting-body formation. In order to develop a robust genetic system for ectopic complementation in *S. aurantiaca*, we developed a plasmid-based system that utilizes site-specific recombination at the *attB* locus. Previous work demonstrated that plasmids containing the *intP-attP* gene integrate into *attB* of *S. aurantiaca* by site-specific recombination with high efficiency (36). In order to facilitate recombination at the *attB* site, a plasmid containing a 2.9-kb fragment of Mx8 DNA was constructed (Fig. 1A). The 2.9-kb fragment contains the *attP* site within the *intP* gene encoding an integrase and the upstream *uoi* gene encoding a putative excisionase that contains the promoter for *intP* (25, 33). Plasmid pSM28 was used to generate mutant SM12 by site-specific recombination (Fig. 1A).

To assess if integration into the *attB* locus has an effect on *S. aurantiaca*, we analyzed mutant SM12 for its ability to form fruiting-body structures. In contrast to *M. xanthus* integrants, the *S. aurantiaca attB* mutant SM12 was unable to form wild-type fruiting bodies (Fig. 2). The fruiting-body structures observed for SM12 are similar to those structures formed transiently during wild-type development (44). However, SM12 cells were still able to produce viable spores, as well as indole-induced spores (not shown); the latter are independent of stalk and sporangiole formation. These data indicate that stalk formation is specifically affected without blocking spore formation. Further analysis of the *trnVD* locus was carried out by integrating a *neo* cassette upstream of *trnV* (SM31), within *trnV* (SM32), and within *trnD* (SM33). All three mutant strains showed no defect during vegetative growth but displayed a block in development similar to that seen for strain SM12 (Fig. 2). These observations indicate that normal expression of *trnVD* is required for fruiting-body formation.

The *attB* locus of *S. aurantiaca* resides within the *trnVD* operon. Analysis revealed that the *S. aurantiaca attB* locus lies within a two-gene operon comprising *trnV* and *trnD*, encoding $tRNA^{Val}$ and $tRNA^{Asp}$, respectively. In contrast, the *attB* locus in *M. xanthus* contains three genes, *trnV*, *trnD1*, and *trnD2* (24) (Fig. 1B). The *trnD* gene of *S. aurantiaca* contains the 17-bp core sequence for site-specific integration near the 3′ end of the gene. The predicted terminus of the *trnV* gene is located 5 bp upstream of the start of the *trnD* gene and contains no core sequence for phage integration. Comparison of the tRNA se-

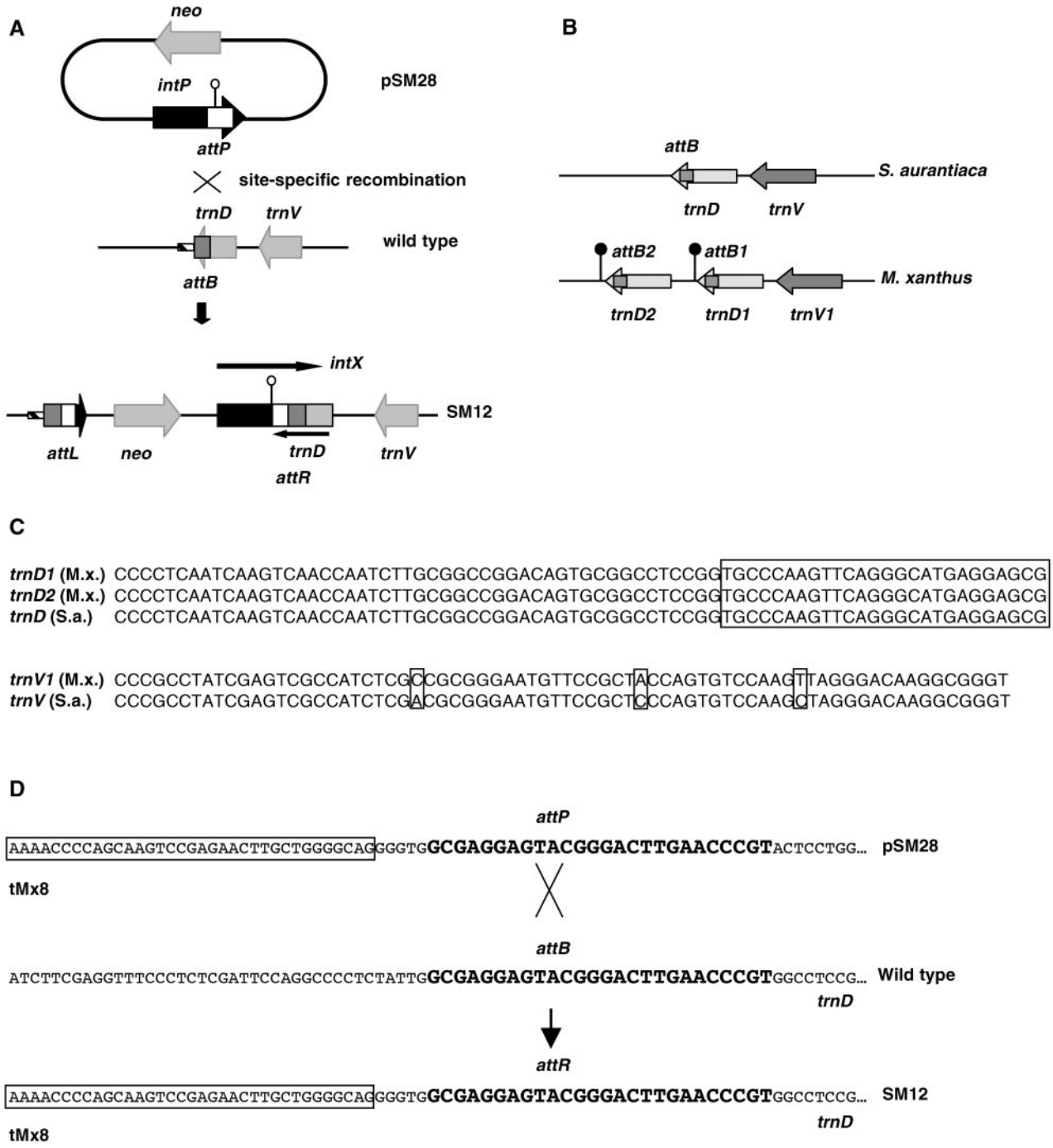

FIG. 1. (A) Model for site-specific recombination into *attB* of *S. aurantiaca*. pSM28 recombines at *attB* within *trnD* in the *S. aurantiaca* chromosome, generating strain SM12. IntP catalyzes site-specific recombination between *attP* (white square) and *attB* (gray square), producing host-phage junctions *attL* and *attR* in strain SM12. Recombination replaces the *trnD* downstream region (striped rectangle) with the Mx8 terminator (t_{Mx8}) sequence (stem-loop) embedded within *intP*. Following recombination, *intP* is shortened to *intX*. (B) Comparison of *S. aurantiaca* and *M. xanthus attB* loci. Both organisms contain one *trnV* gene encoding $tRNA^{Val}$. *M. xanthus* possesses two *trnD* genes encoding $tRNA^{Asp}$, with core sequence for site-specific recombination at the 3′ ends (*attB1* and *attB2*, indicated as gray squares). *S. aurantiaca* contains one *trnD* gene with *attB* (gray square). Terminator structures in *M. xanthus* (stem-loop) are indicated. (C) Sequence alignment of *trnD* and *trnV* genes from *M. xanthus* and *S. aurantiaca*. The core sequence for site-specific recombination at the 3′ ends of the *trnD* genes is marked with a box. The *trnD* sequences shown are identical. Three bases differ between the *trnV* genes of the two species (boxed). (D) The downstream region of *trnD* is altered following integration of pSM28. The *attP* and *attB* sequences are shown in boldface, and the Mx8 terminator (t_{Mx8}) is boxed. Following integration of pSM28, mutant SM12 contains the terminator sequence distal to the core attachment site at the 3′ end of *trnD*.

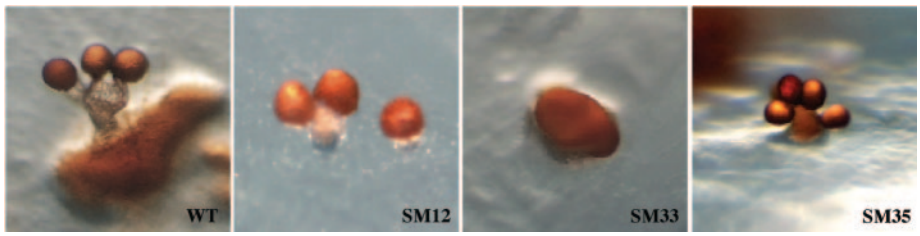

FIG. 2. Fruiting-body formation by *S. aurantiaca* wild-type and mutant strains. Wild-type (WT) fruiting bodies differentiate into branched stalks with sporangioles. Mutants SM12 and SM33 (*trnD*::*neo*) form fungus-like clumps typically observed in the early stages of development by the WT. Strain SM35 (SM12 complemented by a wild-type copy of *trnVD*) restores fruiting-body formation. The photomicrographs were taken at different magnifications due to the various sizes of the aggregates, which range from 10 to 50 μm in diameter.

quences from *S. aurantiaca* and *M. xanthus* showed that the *trnD* genes are identical, while the *trnV* genes differ at three positions (Fig. 1C). This three-base difference does not affect the predicted cloverleaf structure of $tRNA^{Val}$ compared to that of *M. xanthus* (not shown). For the *trnD* genes in *M. xanthus*, terminators with stem-loop secondary structure have been identified (24). Downstream of $tRNA^{Asp}$ and $tRNA^{Val}$ in *S. aurantiaca*, no such stem-loop structures were found (Fig. 1D).

Expression of *trnVD* is developmentally regulated in *S. aurantiaca*. Because mutant SM12 displayed no growth or motility defects but did display a defect in fruiting-body formation, we hypothesized that *trnVD* might be specific for development. In order to determine the timing of expression of the *trnV* and *trnD* genes, a *lacZ* fusion construct was generated with the predicted promoter region for the *trnVD* operon. A fragment containing 563 bp of upstream DNA, *trnV*, and 57 bp of *trnD* (700 bp total) was fused to *lacZ*, resulting in plasmid pSM96. Plasmid pSM96 was electroporated into *S. aurantiaca* and integrated into the genome by a single homologous recombination event. The resulting merodiploid (SM41) contains a 3′-truncated copy of *trnD* fused to *lacZ* under the control of the endogenous promoter (Fig. 3A). β-Galactosidase activity was determined for SM41 under vegetative and developmental conditions (Fig. 3B). The β-galactosidase activity in SM41 cells was easily detected during vegetative growth and increased steadily during development (Fig. 3B). The results allow us to conclude that the *trnVD* locus is expressed vegetatively but is also developmentally upregulated. Furthermore, cells from strain SM41 build wild-type fruiting bodies, indicating that insertion of plasmid pSM96 did not affect stalk formation (not shown). This result indicates that the promoter region for *trnVD* is contained with the 563-bp sequence upstream of *trnV*.

Integration of plasmids into the *S. aurantiaca attB* locus results in replacement of the natural *trnD* downstream region. In order to determine the nature of the defect in stalk formation, we examined the result of integration into the *attB* locus more closely. In *M. xanthus*, both *trnD* genes contain a terminator with stem-loop secondary structure (24). Because the Mx8 *attP* site is located within the coding sequence of the *intP* integrase gene and the *attB* core sequence is located at the 3′ end of the *M. xanthus trnD* genes, recombination at *attB* results in a replacement of the natural terminator for *trnD1* or *trnD2* with the t_{Mx8} terminator (24). In contrast, *trnD* in *S. aurantiaca* has no predicted terminator. Thus, recombination at *attB* with plasmids containing the *intP-attP* sequence introduces the t_{Mx8} terminator at the 3′ end of *trnD* distal to *attB*. The presence of the terminator was verified by sequencing the *attR* host-phage junction in mutant SM12 (Fig. 1D).

Integration at the *attB* locus results in decreased expression of *trnVD* and lack of processing of the 3′ trailer sequence. The presence of a terminator sequence downstream of *trnD* could affect the level of transcription of the *trnVD* locus or block processing of the transcript, thereby generating the developmental defect displayed by mutant SM12. To measure the *trnVD* transcript levels, we performed semiquantitative PCR

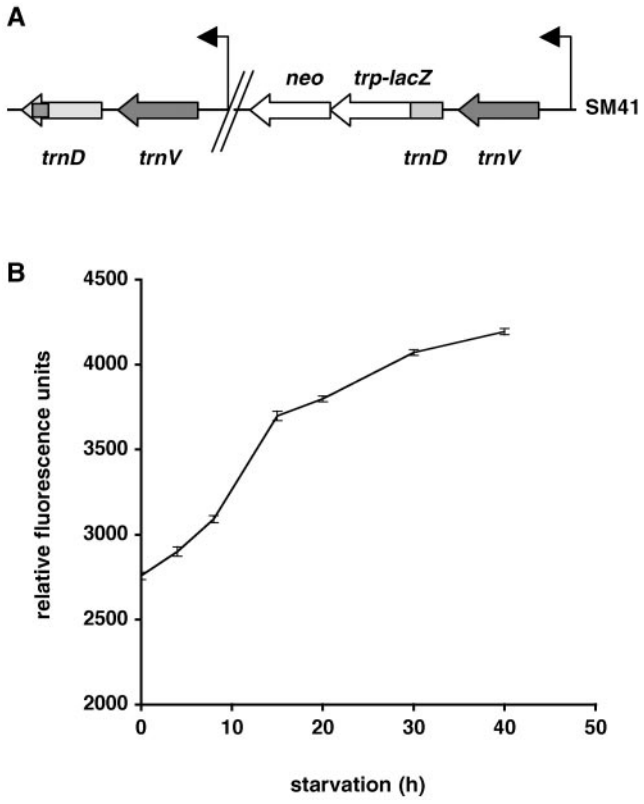

FIG. 3. *trnVD* expression is developmentally regulated. (A) Merodiploid strain SM41 contains a Δ*trp-lacZ* reporter fused to a truncated *trnD* gene. The region upstream of both *trnV* genes is 563 bp and contains a promoter driving expression of the *lacZ* fusion. SM41 forms wild-type fruiting bodies. (B) β-Galactosidase activity was measured by fluorometric detection of 4-MU (see Materials and Methods) from SM41 cell lysates isolated at the times indicated during development.

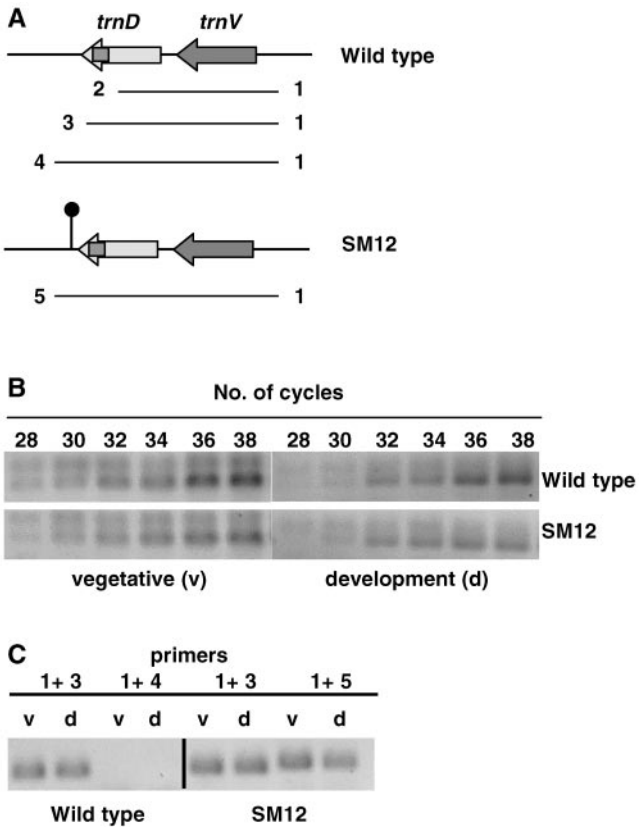

FIG. 4. Analysis of *trnVD* transcripts in WT and SM12 cells. (A) Shown are primer combinations used for PCR amplification of cDNAs produced by reverse transcriptase using total RNA as a template. Primer 1 hybridizes 49 bp upstream of the predicted 5′ end of *trnV*. Primer 2 hybridizes within *trnD*. Primer 3 is identical to the 3′ end of *trnD*. Primer 4 is specific to the region 22 bp downstream of *trnD* in the wild type. Primer 5 is specific to the region 47 bp downstream of *trnD* in mutant SM12. The attachment site, *attB* (gray square), and terminator in SM12 (stem-loop) are shown. (B) Semiquantitative PCR was carried out as described in the text for the wild-type and mutant SM12. Total RNAs (1 μg and 0.5 μg) from vegetative and developmental (24-h) extracts, respectively, were used as templates for reverse transcriptase to generate cDNA in proportion to the *trnVD* transcript level. Primers 1 and 2 were used for these reactions, which were stopped after the number of cycles shown. (C) PCR (36 cycles) of cDNAs was performed with the primer combinations shown. *trnVD* transcript is generated by the wild type and SM12 (1 + 3) and is processed normally by the wild type (1 + 4), but not in mutant SM12 (1 + 5) due to the presence of the 3′ trailer sequence. v, vegetative; d, developmental (24 h).

on cDNAs generated by reverse transcriptase, using total RNAs isolated from wild-type and mutant SM12 cells as templates. Primers 1 and 2 were used for PCR amplification of *trnVD* from either wild-type or mutant SM12 (Fig. 4A). Compared to the wild type, the level of this specific transcript was lower in mutant strain SM12 under both vegetative and starvation conditions (Fig. 4B). This result demonstrates that the change in the *trnD* downstream region in mutant SM12 affects the transcript level of *trnVD*. Furthermore, sequencing of these transcripts confirmed that the amplified product was specific to *trnVD*, indicating that *trnV* and *trnD* compose an operon.

To assess processing of the *trnVD* transcript, PCR from the same cDNA template was performed using various primer combinations specific to either the wild-type or mutant SM12 sequence. Primers 1 and 3 span both *trnV* and *trnD* but do not include any downstream sequence. The results show that both *trnV* and *trnD* are transcribed in both strains (Fig. 4C). Because the downstream region of *trnD* is different for the wild-type and mutant SM12 (Fig. 1D), two distinct downstream primers were used to PCR amplify any unprocessed *trnVD* transcript (Fig. 4A). The results clearly show that no transcript was detectable from wild-type cells using primers 1 and 4, indicating that normal processing of the 3′ trailer sequence of *trnVD* is likely to have occurred. However, transcript was detected from SM12 cells using primers 1 and 5, indicating that the 3′ trailer sequence was not processed properly in this mutant. Therefore, both the level and processing of the *trnVD* transcript are abnormal in mutant SM12 cells.

Development is restored by complementation with *trnVD*. Integration of plasmid pSM28 into the *attB* locus resulted in the formation of abnormal fruiting bodies (Fig. 2). Because the results described above may be due to polar effects on an unidentified gene downstream of *trnVD*, we attempted to complement mutant SM12 by expressing a wild-type copy of the *trnVD* genes, including the promoter and downstream region, from an ectopic locus. Plasmid pSM86 contains a 2.3-kb fragment with *mtaB* encoding a polyketide synthase (40), a *tet* gene for selection, and a 1.2-kb fragment containing the entire *trnVD* locus. Mutant SM12 was transformed with pSM86, generating strain SM35, which was able to form wild-type fruiting bodies (Fig. 2). Thus, the defect displayed by SM12 cells is not due to polar effects on downstream genes.

It is worth noting that pSM86 should integrate at *mtaB* due to the large region of homology. This was found to be the case when pSM86 was introduced into the wild type, generating SM34, which displayed normal fruiting-body formation (not shown). However, pSM86 did not integrate into *mtaB* in mutant SM12 but was found to have integrated at the *trnVD* locus, generating a merodiploid. The site of integration was confirmed by Southern analysis of the complemented SM35 cells. The most likely explanation is that pSM86 integrated by site-specific recombination into *attR* due to the IntX recombinase that should be present in SM12 cells. It was previously demonstrated for *M. xanthus* that the *intP* gene (containing *attP* on the integrating plasmid) is disrupted following integration at *attB*. Integration generates a shorter open reading frame designated *intX* and encodes an integrase with a new C terminus and lower activity (24, 25, 43). We have not assayed for IntX activity in SM12 cells, although the shorter *intX* open reading frame is present.

***trnVD* mutants display abnormal protein production.** The disruption of *trnVD* expression and processing observed in mutant SM12 results in the formation of structures that are usually transiently observed during wild-type development (Fig. 2) (44). Because *trnV* and *trnD* encode $tRNA^{Val}$ and $tRNA^{Asp}$, respectively, any defect in processing *trnVD* should affect translation of developmentally regulated proteins. To investigate this hypothesis, wild-type and mutant SM12 cells were analyzed for protein production during vegetative growth and development. Extracts were subjected to 2-D electrophoresis. A change in the protein production pattern was visible by comparing the two strains during development (Fig. 5),

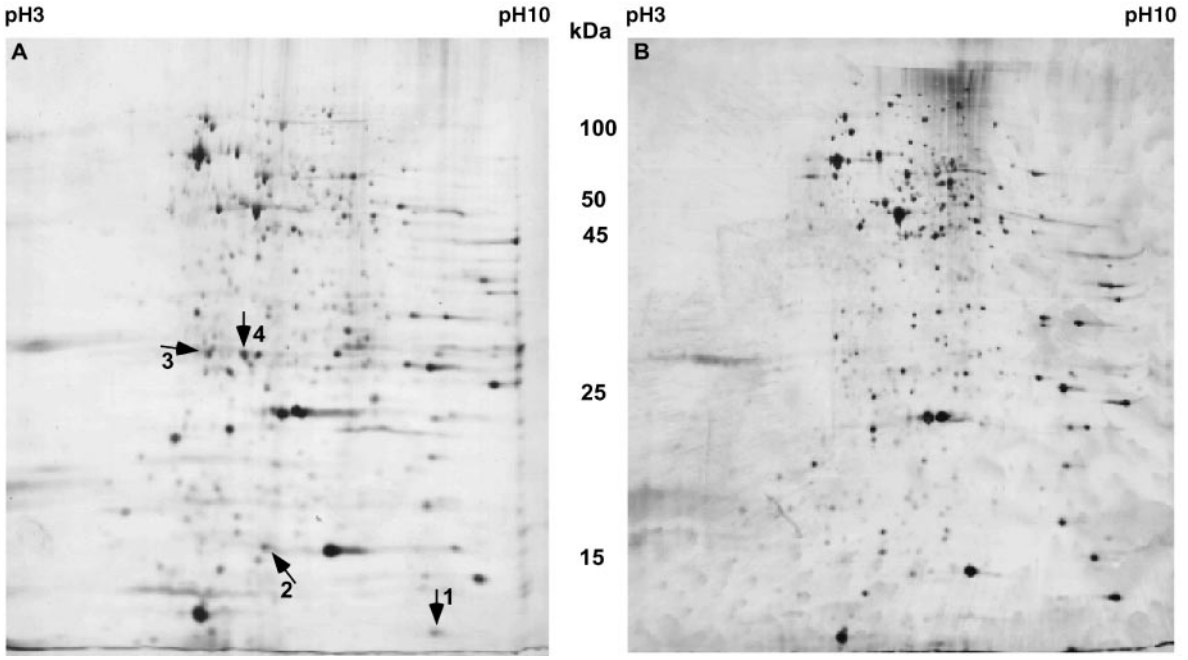

FIG. 5. Analysis of protein production during development by WT and SM12 cells. Cells were harvested after 16 h of starvation and analyzed by 2-D gel electrophoresis as described in Materials and Methods. (A) WT and (B) SM12 cell extracts are shown. The arrows indicate spots that were produced by the wild type and that are absent from SM12 cells. Sequences of the four peptides showed no similarity to any protein in the database. Relative masses are shown.

but not during vegetative growth (data not shown). Several proteins were differentially expressed in the wild-type and mutant SM12 cells. Peptide sequences were determined for those spots found in the wild type but absent in mutant SM12. BLAST searches against the nonredundant database revealed no homology to known proteins, including those of the closely related *M. xanthus*. These data support a model that requires $tRNA^{Val}$ and $tRNA^{Asp}$, encoded by *trnVD*, for developmental-gene expression in *S. aurantiaca*.

DISCUSSION

In order to develop a genetic system for *Stigmatella aurantiaca*, we took advantage of previous work by Magrini and colleagues (24, 25). Plasmids containing the *intP-attP* gene of phage Mx8 are known to recombine into the *attB* site of *M. xanthus* with high efficiency. Because no myxophages are known to infect *S. aurantiaca*, we generated a plasmid containing the Mx8 *intP-attP* gene for integration into the *S. aurantiaca* chromosome. Integration of plasmids containing the *intP-attP* gene was found to occur at the preferred unique *attB* site, resulting in *S. aurantiaca* derivatives that were genetically stable during development (36).

The *attB* locus of *S. aurantiaca* was cloned and sequenced and found to span two tRNA genes, *trnV* and *trnD*, encoding $tRNA^{Val}$ and $tRNA^{Asp}$, respectively. Analysis indicated that *trnV* and *trnD* are cotranscribed and compose an operon, similar to what is observed for clustered tRNA genes in other bacteria (5). No other open reading frames were identified as part of the *trnVD* operon. The *trnD* gene contains the 17-bp core sequence for site-specific recombination at its 3′ end. In *M. xanthus*, the *attB* region contains two *trnD* genes and one *trnV* gene (24). Each *M. xanthus trnD* gene contains the core sequence at the 3′ end. The sequences for the *trnD* genes in *M. xanthus* and *S. aurantiaca* are identical, while the *trnV* gene of *S. aurantiaca* differs from that of *M. xanthus* by only three bases (Fig. 1C). The tRNA genes from *S. aurantiaca* do not code for the CCA acceptor stems, indicating that the CCA acceptor stems are added enzymatically during tRNA maturation, as described for other class II tRNAs (6). The requirements for site-specific recombination identified by Gabriel et al. (8) are fulfilled in *S. aurantiaca*.

In contrast to what has been previously described for *M. xanthus*, site-specific recombination into *attB* of *S. aurantiaca* resulted in the formation of abnormal fruiting bodies that consisted primarily of small clumps instead of the complex tree-like structures formed by the wild type (Fig. 2). The intermediate structures formed by the insertion mutants are transiently formed by the wild type during fruiting-body formation (44). We were able to restore wild-type fruiting-body formation in mutant SM12 by complementation with the full-length *attB* locus, thereby allowing us to rule out any polar effects. The defect displayed by mutant SM12 cells is therefore attributable to the altered 3′ downstream region of the *trnVD* operon. Semiquantitative PCR analysis of cDNA derived from *trnVD* precursor RNA indicated that the operon was expressed at a lower level both vegetatively and developmentally in mutant SM12 relative to that from the wild type (Fig. 4B). Furthermore, the 3′ trailer sequence for *trnVD* was detectable in mutant SM12 cells, indicating that normal processing of the precursor tRNA did not occur as efficiently as in the wild type, where a precursor transcript was not detectable under any

conditions. These results allow us to conclude that altering the downstream region of the *trnVD* transcript affects the overall levels of the transcript, as well as its maturation. The 3′ trailer sequence usually contains recognition sites for 3′ trailer-trimming endoribonucleases and exoribonucleases (5). The altered 3′ *trnVD* region in mutant SM12 could inhibit the processing of the 3′ trailer that is required for subsequent addition of the CCA acceptor stem. Altered processing of *trnV* and/or *trnD* would therefore prevent charging of these tRNAs with the appropriate amino acids and affect translation of message with codons dependent upon these tRNAs.

Analysis of a merodiploid strain containing a Δ*trp-lacZ* reporter fusion with *trnD* (SM41) (Fig. 3A) indicated that the *trnVD* locus is expressed constitutively during vegetative growth and upregulated during starvation. This merodiploid strain contains the *trnVD* promoter and was able to form wild-type fruiting bodies, indicating that β-galactosidase activity in SM41 reflects wild-type expression of *trnVD*. Together, the data allow us to conclude that the *trnVD* locus is specifically required to complete the developmental program in *S. aurantiaca*. The differences in protein production displayed by the mutant SM12 relative to the wild type, as observed by 2-D analysis, support this conclusion. It is likely that the *trnVD* locus is required for synthesis of proteins dedicated to stalk formation in *S. aurantiaca*.

tRNA genes are a preferred target for site-specific integration in various organisms (2, 32). In general, the coding sequence of the tRNA genes is not altered after recombination, because these genes are often essential and unique. However, in *Streptomyces lividans*, a gene encoding $tRNA^{Tyr}$ overlaps part of the *attB* site and is essential for cell viability, as it could only be deleted when a second copy was present in the chromosome (45). In *Streptomyces coelicolor*, it has been shown that the formation of aerial hyphae is dependent upon a specific $tRNA^{Leu}$ encoded by the *bldA* gene, indicating the usage of a rare codon specifically required for development (3, 19, 20). Likewise, *S. aurantiaca* requires the *trnVD* locus specifically for stalk formation and normal fruiting-body formation. Thus, *trnVD* defines a new developmental checkpoint for the developmental program in *S. aurantiaca*.

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